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BRIEF COMMUNICATION

Lack of resistance-associated mutations in UL54 and UL97 genes of circulating Cytomegalovirus strains isolated in a medical center in Taiwan

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Human cytomegalovirus (HCMV) is a large DNA virus and a member of the betaherpesvirus family. HCMV infection is extremely common in human populations and can cause severe diseases in immunocompromised hosts. Ganciclovir is the most widely used antiviral drug for cytomegalovirus infection and works by blocking the amplification of HCMV. HCMV strains resistant to ganciclovir have been detected in recent decades and mainly result from mutations in UL97 (protein kinase) and UL54 (DNA polymerase) genes. In order to understand the prevalence of resistance of HCMV in Taiwan, we studied 40 clinical isolates to detect the mutations of UL97 and UL54 that might be related to resistance. The results showed that no mutation known to cause ganciclovir resistance was detected in any strain, but some polymorphisms (N685S, A688V, A885T, N898D in UL54; D605E in UL97) were frequently observed. Our results suggest that resistant HCMV strains are not prevalent in Taiwan.

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous virus and infects the great majority of humans early in their lives.[1] Being a human herpesvirus, HCMV is capable of establishing life-long latency after primary infection.[1,2] Reactivation

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of HCMV is not a rare event in the lifetime of infected individuals. HCMV infection is a particular problem among immunocompromised patients, such as those with AIDS, recipients of bone marrow and organ transplants, and those receiving immunosuppressive or chemotherapeutic agents.[3,4] Currently, three effective antiviral agents (ganciclovir, foscarnet, and cidofovir) are available to suppress HCMV replication, by suppressing DNA polymerase (pUL54) activity.[5,6] Ganciclovir has been widely used clinically for HCMV infections.[7] However, development of drug-resistance in HCMV has been frequently observed and becomes a clinical concern after long-term use of antiviral treatment.[8] Resistance of HCMV can be determined by phenotype or genotype assay.[9] Phenotype assay is cumbersome whereas genotype assay is faster and easier to perform. However, although genotype assay is often able to assist prompt diagnosis, enabling treatment to be started quickly, the mutations detected through genotype assay may be hard to interpret. The mutations may be only polymorphisms (sequence variants *not* reducing drug susceptibility) or actual resistance-conferring mutations. Only marker transfer experiments enable discrimination between polymorphisms and resistance mutations.[9,10]

Ganciclovir is phosphorylated by CMV protein kinase (pUL97) initially, and then undergoes two phosphorylation steps by cellular kinase.[5,6] Resistance of CMV to ganciclovir comes from mutations in two viral genes, namely UL97 and UL54. Mutations in the UL97 gene lead to loss of the phosphorylation function of the UL97 gene product, thus impairing the first step of ganciclovir phosphorylation.[9,10] Ganciclovir, foscarnet, and cidofovir exert antiviral effects through suppression of HCMV DNA polymerase function; hence, mutations in the DNA polymerase gene (UL54) could result in resistance to all the three agents.[11–13] Antiviral therapy and prophylaxis against HCMV infection has been used in Taiwan for decades but the frequency of antiviral resistance among Taiwan HCMV strains is unknown. This study aimed to understand the mutations in UL97 and UL54 genes in HCMV strains circulating in Taiwan.

Materials and methods

Virus and nucleic acid extraction

We collected 40 HCMV isolates from clinical specimens using a human fibroblast cell line in the National Taiwan University Hospital during 2009 and 2010. The complete nucleic acid was extracted from the supernatant of the virus-infected cell culture by MagNA Pure LC 2.0 (Roche Diagnostics, German).

Genotypic analysis of the UL97 and UL54 genes

Amplification of antiviral resistance regions of UL97 (domains VI, VIII, and IX) and UL54 (domains IV to V)[11,12] using nested polymerase chain reactions (nested-PCR), which contained 35 cycles of denaturation for 40 seconds at 95 °C, annealing for 40 seconds at 57.5 °C, and elongation for 75 seconds at 72 °C. The first round primers for the UL97 region (codons 382-614) were outer-UL97-F (5'-ggACATgAgCgACgAgAgCT-3') and outer-UL97-R (5'-gTACgCgACACgAggACATC-3') and second round primers were inner-UL97-F (5'-ggTgCTCACggTCTggATgT-3') and inner-UL97-R (5'-AgACAggCgCCgTAgCTCAT-3'). For amplification of UL54 (codons 340-989), the first round primers of the front region were outer-UL54-A-F (5'-ATCggCggATCACCACgTTC-3') and outer-UL54-A-R (5'-gTTCTCTAgCgTgACgCTgTAT-3'), and the second round primers were inner-UL54-A-F (5'-ATTCA gATCTCgTgCgTgTgCT-3') and inner-UL54-A-R (5'-TgRgCCAT gATgATggAAgg-3'). The first round primers of the rear region of UL54 were outer-UL54-B-F (5'-CCAggTAggCCgT TACTgTCT-3') and outer-UL54-B-R (5'-ACACggTgCAggTA CAgATCgT-3'), and the second round primers were inner-UL54-B-F (5'-TggTgCgCgATCTgTTCAACAC-3') and inner-UL54-B-R (5'-gCTTCCgAgACCTCgCgATCCT-3').

DNA sequencing and data analysis

PCR products were recovered by using a Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan), and sequenced by automated DNA sequencing (ABI 3730). The nucleotide acid sequences were compared with AD169 using EBI ClustalW2, version 2.0 (Cambridge, UK) and GeneDoc, version 2.6, software (http://www.psc.edu/biomed/genedoc).

Results

The HCMV were isolated from 40 clinical specimens, consisting of 20 throat swabs, 7 blood samples, 5 sputa, 4 urine samples and 4 aqueous humors (Table 1). To be representative, the HCMV clinical isolates were collected from 25 inpatients and 15 outpatients. The inpatients had been diagnosed with various diseases, and nine of these patients were receiving ganciclovir following transplantation or for serious HCMV infection. HCMV strains were also isolated from 15 outpatients who had less severe illnesses. The UL54 and UL97 genes were amplified and sequenced. There were no known mutations conferring ganciclovir resistance in either the inpatient or outpatient isolates. However, we detected some common polymorphisms in local HCMV strains. The most common polymorphism in UL97 was D605E, observed in 28 of the 40 (68%) isolates. Four known UL54 polymorphisms (N685S, A688V, A885T, N898D)[14] were also frequently detected in 29 of the 40 (73%) isolates.

Of the eight transplant recipients (patients 25, 28, 29, 30,31, 37, 38, and 40) from whom clinical isolates were collected, four were receiving ganciclovir (patients 29, 31, 37, and 40). No distinct polymorphism was found in the transplant recipients, except for four changes (K409 M, T690A, S694A in UL54 and G391S in UL97) detected in patients 28, 31, and 40. On the other hand, nine patients received ganciclovir for serious HCMV infection (patients 27, 29, 31, 34, 35, 37, 38, 39, and 40), and seven of them revealed unusual variations (see Table 1).

In order to assess whether the ganciclovir-resistant strains were widespread, we collected 15 HCMV strains from outpatients who were community dwellers without obvious immune suppression. Thirteen of these outpatient isolates (87%) had polymorphisms (see Table 1).

Table 1 Sequence polymorphism in antiviral regions of UL54 and UL97 compared with AD169 strain and the patient/specimen type and period of ganciclovir treatment.

Patient ID	UL54			UL97			Ganciclovir treatment (days)	Sample source[e]	Diagnosis[f]	Specimen type[g]
	Ganciclovir resistant[a]	Polymorphism[b]	Unknown[c]	Ganciclovir resistant[a]	Polymorphism[d]	Unknown[c]				
1	–	N685S, A688 V, A885 T, N898D	–	–	–	–	–	O. C.	Panuveitis	aq. H
2	–	N685S, A688 V, A885 T, N898D	–	–	–	–	–	O. C.	Panuveitis	aq. H
3	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	O. C.	Panuveitis	aq. H
4	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	O. C.	Panuveitis	aq. H
5	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	O. C.	URI	TH
6	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	O. C.	URI	TH
7	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	O. C.	URI	TH
8	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	O. C.	URI	TH
9	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	O. C.	Epilepsy, URI	U
10	–	N685S, A688 V, A885 T, N898D	L957P	–	D605E	–	–	O. C.	Croup	TH
11	–	N685S, A885 T, G874R, N898D	T690A	–	D605E	–	–	O. C.	Congenital CMV infection	B
12	–	S655L, N685S, G874R, A885 T, N898D	–	–	D605E	A590 V	–	O. C.	Pneumonia	U
13	–	S655L, N685S, G874R, A885 T, N898D	S646F, G678S	–	D605E	–	–	O. C.	Cholestasis in newborn	TH
14	–	S655L, A688 V, G874R, A885 T, N898D	A472 T	–	D605E	A477 V	–	O. C.	AOM	TH
15	–	N685S, A688 V, G874R, A885 T, N898D	–	–	D605E	–	–	O. C.	URI	TH
16	–	N685S, A688 V, A885 T, N898D	–	–	–	–	–	I. C.	ALL, Klebsiella pneumonia	Sp
17	–	N685S, A688 V, A885 T, N898D	–	–	–	–	–	I. C.	TOF, influenza	TH
18	–	N685S, A688 V, A885 T, N898D	–	–	–	–	–	I. C.	TOF	TH
19	–	N685S, A688 V, A885 T, N898D	–	–	–	–	–	I. C.	OHT/DCM	B
20	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	I. C.	Bronchopneumonia	TH
21	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	I. C.	Acute bronchiolitis	TH
22	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	I. C.	AIDS, interstitial pneumonitis	TH
23	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	I. C.	Acute bronchiolitis	TH
24	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	I. C.	Multiple myeloma	TH
25	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	I. C.	Liver transplantation/ chronic HCV infection with liver cirrhosis	B
26	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	–	I. C.	Pneumonia	TH
27	–	N685S, A688 V, A885 T, N898D	–	–	D605E	–	42	I. C.	Congenital CMV infection	U
28	–	N685S, A688 V, A885 T, N898D	–	–	–	G391S	–	I. C.	PBSCT/ALL	B
29	–	N685S, A688 V, A885 T, N898D	–	–	–	L568 G	25	I. C.	CBT/CMV pneumonitis	Sp
30	–	N685S, A688 V, G874R, A885 T, N898D	–	–	D605E	–	–	I. C.	PBSCT/multiple myeloma	TH

31	—	N685S, A688 V, A885 T, N898D	K409 M	—	D605E	—	10	I. C.	PBSCT/ myelodysplastic syndrome	TH
32	—	N685S, A688 V, N898D	K409 M	—	D605E	—	—	I. C.	Infantile spasm, enterovirus meningitis	TH
33	—	N685S, A688 V, A885 T, N898D	668—680 deletion	—	D605E	—	—	I. C.	AOM	U
34	—	N685S, A688 V, A885 T, N898D	M828E insertion	—	—	—	14	I. C.	CMV hepatitis	B
35	—	N685S, A885 T, N898D	M828E insertion	—	D605E	—	20	I. C.	ALL, CMV pneumonitis	Sp
36	—	A885 T, S897L, N898D	—	—	—	—	—	I. C.	Lung cancer, pneumonia	TH
37	—	S655L, N685S, G874R, A885 T, N898D	—	—	—	—	10	I. C.	sBMT/Beta-thalassemia major	Sp
38	—	N685S, A688 V, A885 T, N898D	—	—	D605E	E581A, L583 W	10	I. C.	ALL, pneumonia	B
39	—	N685S, A688 V, A885 T, N898D	P642S, G672S	—	D605E	—	42	I. C.	Congenital CMV infection	B
40	—	N685S, A885 T, G874R, N898D	T690A, S694A	—	—	—	10	I. C.	PBSCT/JMML	Sp

[a] The known UL97 and UL54 GCV- resistance mutation were found.[14,15]
[b] Natural polymorphism among HCMV.[16,17]
[c] UL54 variations have not been reported previously.[8,16]
[d] The mutation D605E of UL97 is regarded as a natural polymorphism.[18]
[e] Sample source: outpatient clinic (O. C.)/inpatient clinic (I. C.)
[f] AIDS = acquired immunodeficiency syndrome; ALL = acute lymphoid leukemia; AOM = acute otitis media; CBT = cord blood transplantation; DCM = dilated cardiomyopathy; JMML = juvenile myelomonocytic leukemia; OHT = orthotopic heart transplantation; PBSCT = peripheral blood stem cell transplantation; sBMT = sibling bone marrow transplantation; TOF = tetralogy of Fallot; URI = upper respiratory tract infection.
[g] aq. H = aqueous humor; B = blood; Sp = sputum; TH = throat swab; U = urine.

Discussion

This is the first study to systemically examine the sequence variation in UL54 and UL97 in cytomegalovirus strains in Taiwan. Many mutations were found, but none of those identified in the 40 samples were definitely associated with ganciclovir resistance (see Table 1); most of them were polymorphisms, including D605E in UL97 and the common combination of N685S, A688V, A885T, N898D in UL54. These polymorphisms were found in high frequency both in inpatients and outpatient isolates. D605E in UL97 was commonly found in our study. Although the UL97 mutation at codon 605 (D605E) was regarded as a natural variation or polymorphism,[18] recent studies have revealed that double mutations A594P/D605E and M460 V/D605E have conferred ganciclovir resistance.[19,20] We believe that the high frequency of D605E variation might easily give rise to ganciclovir-resistant HCMV in the future.

There were some unusual mutations, including nine in UL54 (K409 M, A472 T, P642S, S646F, G672S, G678S, T690A, S694A, and L957P) and six in UL97 (G391S, A477 V, L568 G, E581A, L583 W, and A590 V).[8,16] The frequency of these mutations was 11/40 (28%) isolates in UL54 and 5/40 (13%) in UL97. Notably, five of the nine (56%) patients who were receiving ganciclovir harbored the unusual UL54 mutations in their HCMV isolates. In contrast, only six among 31 (19%) patients without concurrent ganciclovir had mutations in UL54, irrespective of their diagnosis. There were 2/9 (22%) patients with ganciclovir treatment and 3/31 (10%) patients without ganciclovir treatment who had unusual mutations in UL97. The mutations in UL54 and UL97 seemed more frequently found in the patients who were receiving ganciclovir. We could not infer what influence these mutations might have had concerning resistance to ganciclovir without using phenotypic analysis to calculate the 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) for ganciclovir. When judging the possibility of ganciclovir resistance, the combination of genotypic and phenotypic analysis is necessary.[13]

A proportion of the HCMV strains tested were derived from immunocompromised patients who may have been taking antiviral drugs. We expected to detect resistance-associated mutations in these HCMV strains but failed to achieve that aim. Nevertheless, some polymorphisms and unusual mutations were found in our study. Whether these variations predispose HCMV isolated in Taiwan to drug resistance needs further clarification. Therefore, continuous monitoring of HCMV in Taiwan is needed to understand the antiviral resistance status of the virus in Taiwan and to guide its clinical management.

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